Topical Retinoic Acid Enhances, and a Dark Tan Protects, from Subedemal Solar-Simulated Photocarcinogenesis

Gary M. Halliday, Bryan O. Robertson, and Ross St. C. Barnetson

Department of Medicine (Dermatology), University of Sydney at Royal Prince Alfred Hospital, Sydney, New South Wales, Australia

Studies into the effects of topical retinoic acid on photocarcinogenesis have yielded ambiguous findings. This may be due to different experimental protocols and ultraviolet spectra. Retinoic acid is commonly used for a range of dermatologic conditions, and therefore it is important to resolve whether it affects skin tumor formation. To address this issue we used a protocol to mimic as closely as possible human use of retinoic acid. Two mouse strains were used: Skh:HR-1 (albino) and Skh:HR-2 (lightly pigmented). The pigmented mice more closely resemble Caucasian skin as they develop a light tan in response to ultraviolet radiation. This tan is greatly augmented by retinoic acid. As these are congenic mice, any differences can be attributed to the development of a tan. Mice were exposed to solar-simulated ultraviolet radiation, followed by treatment with 0.05% retinoic acid. This modeled exposure to sunlight during the day followed by retinoic acid treatment and a night-time period in the absence of sunlight. As it is recommended that ultraviolet exposure is minimized when using topical retinoic acid, the mice were only exposed to one-third of minimal edemal dose of ultraviolet radiation per day. This retinoic acid protocol augmented photocarcinogenesis. Retinoic acid decreased the latency period, reduced the probability that a mouse would survive without a tumor, and increased the number of tumors per mouse. All tumors induced were squamous cell carcinomas, and the skin between the tumors on mice treated with retinoic acid was found to contain carcinoma *in situ* upon histologic diagnosis. The light tan of the solvent-treated pigmented mice did not provide any protection, whereas the dark tan, which developed in Skh:HR-2 mice in response to retinoic acid, reduced photocarcinogenesis but did not overcome the augmenting effect of retinoic acid. Thus, using this experimental design, topical retinoic acid augmented photocarcinogenesis, and the ability to develop a dark but not light tan provided some, but limited, protection. ***Key words: retinoids/squamous cell carcinoma/sunlight/ultraviolet radiation. J Invest Dermatol 114:923–927, 2000***

Topical all-*trans* retinoic acid (RA) is now commonly used for treatment of diverse dermatologic conditions, and a large number of experimental and clinical studies have shown that retinoids modulate carcinogenesis in a variety of systems (reviewed by Chen and De Luca, 1995). Ultraviolet radiation (UVR) is the prime cause of skin cancer in humans (English *et al*, 1997), and considering the widespread use of RA, it is important to determine whether or not topical RA affects UVR-induced skin carcinogenesis. Topical RA has been reported to reduce (Epstein and Grekin, 1981; Connor *et al*, 1983), enhance (Epstein, 1977; Forbes *et al*, 1979), and have no effect (Kligman and Kligman, 1981; Kligman and Crosby, 1996) on UVR-induced carcinogenesis. Thus the issue of whether or not topical RA affects photocarcinogenesis remains an unresolved and controversial issue in dermatology.

We have established a model of UVR-induced skin carcinogenesis in mice, where Skh:HR-1 hairless albino mice or Skh:HR-2 hairless pigmented mice are exposed to UVR that has a spectrum closely approximating that of sunlight. These two mouse strains are congenic, so that any differences can be attributed to the ability of the Skh:HR-2 mice to develop a tan. We have previously shown that topical RA augments UVR-induced melanogenesis in these mice (Ho *et al*, 1992), due to enhanced activation of melanocytes (Welsh *et al*, 1999). In this model, mice exposed to low dose subedemal UVR initially develop small scaly regions that clinically resemble solar keratosis, followed later by squamous cell carcinomas (SCCs). This closely resembles SCC development in humans. Humans apply RA at night and therefore have a period of time between application and sun exposure. We therefore designed a protocol to mimic human use of RA. Mice were treated with 0.05% RA, the same concentration as is commonly used by human patients, and then exposed to UVR the following day. As sun exposure is minimized when using RA, the mice were exposed daily to low doses of UVR, one-third of the minimal edemal dose (MED). Whereas RA alone was not carcinogenic, it enhanced photocarcinogenesis. The light tan of the Skh:HR-2 mice did not provide any protection from carcinogenesis, whereas the dark tan that developed in the RA-treated UV-irradiated Skh:HR-2 mice did provide some protection, but not sufficiently to overcome the enhancing effect of RA.

Manuscript received April 16, 1999; revised October 21, 1999; accepted for publication November 3, 1999.

Reprint requests to: Associate Professor Gary Halliday, Department of Dermatology, Blackburn Building D06, University of Sydney, Sydney, NSW, Australia 2006. Email: garyh@med.usyd.edu.au.

Abbreviations: MED, minimal edemal dose; RA, retinoic acid.

MATERIALS AND METHODS

Mice Skh:HR-1 and Skh:HR-2 mice are inbred and hairless. Skh:HR-1 are albino, whereas Skh:HR-2 are lightly pigmented and capable of developing a tan in response to UVR. They originated from the Skin Cancer and Photobiology Unit, Temple University, Philadelphia, PA. The Skh:HR-2 mice have been backcrossed with the Skh:HR-1 mice for more than 20 generations by Dr. Reeve (Department of Veterinary Pathology, University of Sydney, Australia), selecting for lightly pigmented mice capable of developing a tan in response to UVR (Reeve *et al*, 1990). Thus these mice are congenic, differing only in ability to produce melanin, and any differences between these strains can be attributed to pigmentation. Female mice, 9–11 wk old, were used in all experiments and were purchased from the Department of Veterinary Pathology, University of Sydney, Australia. Mice were grouped six per cage, with free access to water and standard mouse pellets (Standard Rat and Mouse Cubes, Doust and Rabbidge, Sydney, Australia).

Retinoic acid Mice were treated topically on their backs with RA type XX (Sigma, St Louis, MO) dissolved at 0.5 mg per ml in a solvent consisting of ethanol, dimethylsulfoxide, and acetone (1:1:6, vol/vol/vol). The RA solution was stored in aliquots at –80°C in the dark. Twenty microliters (34 nmol) was delivered per mouse per treatment as we have described previously (Ho *et al*, 1992). Control mice received solvent alone. Mice were treated with RA immediately after each UVR exposure, so that there was a 24 h period between RA and the next UV exposure on the following day (this period was longer when mice were not irradiated on the following day). For the first 4 wk mice were treated 5 d per wk (Monday to Friday) with UV followed immediately by RA. After this time treatments were reduced to 3 d per wk (Monday, Wednesday, Friday) for the duration of the experiment.

Ultraviolet irradiation Mice were irradiated unrestrained in their cages, 30 cm from the light source. Simulated solar UV radiation was provided by a bank of six 100 W cosmolux RA plus A1-14-100W UVA tubes flanking a single central LSFS72T12 UVB tube housed in a planar arrangement in a reflective batten. The spectral output of his light source was designed to simulate the solar spectrum as closely as possible and has been published previously (Bestak *et al*, 1995). Radiation was filtered through a 0.5 mm layer of cellulose triacetate film (Kodacel; Eastman Chemical Products, Kingsport, TN) which reduced radiation sharply below 295 nm and eliminated all wavelengths below 285 nm, and therefore all UVC. The integrated irradiance of the solar-simulated light source was 3.4×10^{-3} W per cm^2 UVA (320–400 nm) and 1.7×10^{-4} W per cm^2 UVB (285–320 nm). Output of the bank of tubes was monitored using an IL 1350 radiometer (International Light, Newburyport, MA), with an SED 038 UVA and an SED 240 UVB detector. The emission spectra were measured at the Commonwealth Scientific and Industrial Research Organisation (Australia).

Mice were exposed initially to 102 mJ per cm^2 UVB with 2040 mJ per cm^2 UVA per d, which is 33% of the MED for these mice with this light source. The exposures were increased by 20% per wk for the first 4 wk, and then remained at this level for the duration of the experiments. Mice were irradiated 5 d per wk (Monday to Friday) for the duration of the experiments.

Pigmentation Skin color (pigmentation) was measured in each mouse with a reflectance melanin meter (Dia-Stron, Hampshire, U.K.) as we have described previously (Damian *et al*, 1997). This spectrometer contains a tungsten–halogen light source, which shines white light onto the skin via a fiber-optic probe. Light scattered from within the skin is then collected by the probe and analyzed by two narrow-band interference filters at 632 nm (for melanin absorption) and 905 nm (the reference signal). In this way the instrument generates a melanin index that increases with increasing skin pigmentation.

Statistics Tumor incidence, expressed as mouse survival data, was analyzed using the Logrank (Mantel–Cox) test. Tumor multiplicity was analyzed by analysis of variance (Fisher's PLSD). A p value of less than 0.01 was regarded as significant.

RESULTS

These experiments were performed twice on groups of 10 mice, with similar results. The two experiments were then pooled to give 20 mice per group. Tumors greater than 2 mm diameter were counted. Initially, the RA increased desquamation and caused some mild inflammation. This subsided after a few weeks of treatment.

RA augments UV-induced melanogenesis Reflectance spectroscopy of skin pigmentation showed the higher level of melanin in the skin of Skh:HR-2 mice compared with Skh:HR-1 mice prior to irradiation or treatment with RA (Wk 0, **Fig 1**). Skh:HR-2 mice developed an increasing but light tan in response to UV. RA alone, in the absence of UVR, also induced a tan, which was slower to develop than in the UV-irradiated mice but increased with each treatment and eventually overtook the UV-irradiated group. Skh:HR-2 mice treated with both UVR and RA developed considerably deeper pigmentation than the other groups of Skh:HR-2 mice. In contrast, the congenic albino strain Skh:HR-1 mice treated with UVR in combination with RA did not have an augmented melanin index up to about week 10. At later times, however, these mice did have an increased melanin index. This appeared to be caused by increased skin thickening and the development of multiple skin lesions, as these mice did not develop a visually observable tan. **Figure 2** shows the deeper pigmentation of the UVR plus RA treated Skh:HR-2 mice and the increased hyperkeratosis of the UVR plus RA treated Skh:HR-1 mice. Thus Skh:HR-2 mice treated with RA plus UVR had a very dark tan, whereas Skh:HR-1 mice treated with RA plus UVR were untanned, and the Skh:HR-2 mice exposed to UVR only had a light tan compared with the untanned Skh:HR-1 mice.

Tumor incidence The percentage of mice in each group surviving without a tumor is shown in **Fig 3**. No mice in the groups treated with RA in the absence of UVR developed any tumors. Albino Skh:HR-1 mice treated with solvent and UVR started to develop SCCs by week 18 of treatment, with 50% of mice having tumors by week 21. RA significantly reduced the probability of survival without a tumor, with tumors commencing to appear 3 wk earlier at week 15, and 50% incidence at week 17. Skh:HR-2 mice, which developed a light tan in response to UVR (**Fig 1**), showed no significant difference in probability of survival without a tumor from the albino mice (**Figs 2**, **3**). The first tumors appeared at week 19, with 50% incidence by week 21. When treated with RA and UVR, the Skh:HR-2 mice developed a dark tan (**Fig 1**), and the time before appearance of skin tumors was significantly reduced. Tumors first appeared by week 18, and reached 50% incidence at week 19. These mice had a significantly increased probability of survival ($p < 0.001$), however, compared with similarly treated albino mice. Thus RA significantly increased

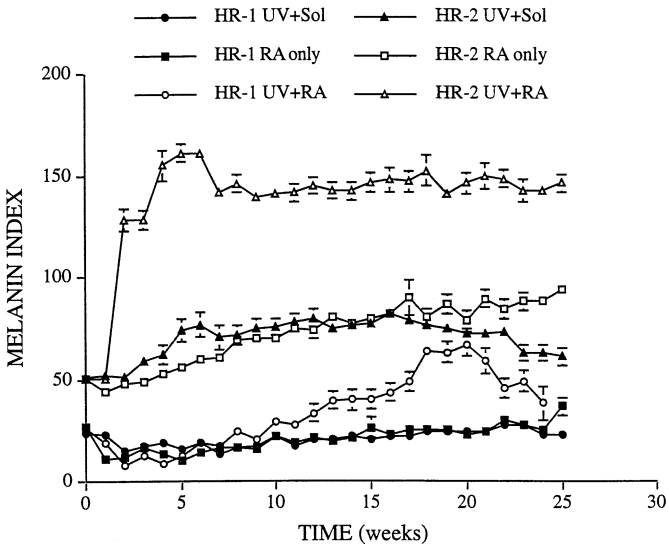

Figure 1. Skh:HR-2 mice but not Skh:HR-1 mice develop increased pigmentation in response to UVR. Groups of 20 mice were exposed to UVR plus solvent (Sol) or RA, or to RA alone, for 25 wk. The melanin index was measured by reflectance spectroscopy at weekly intervals. Mean and SEM are shown; where the SEM cannot be seen, it is too small to be obvious.

tumor incidence in both strains, and the light tan of the UV-irradiated Skh:HR-2 mice did not offer any protection from carcinogenesis. The dark tan of the RA-treated UV-irradiated mice, however, provided protection from skin cancer but was unable to overcome the increase in tumorigenesis caused by the RA.

Figure 2. Topical RA enhances UVR-induced carcinogenesis in Skh:HR-1 and Skh:HR-2 mice. Mice were treated with RA only, UVR and solvent (UVR + Sol), or UVR and RA (UVR + RA) for 20 or 24 wk. Group A refers to the fact that these mice were from the first round of experiments (two rounds were pooled into final results).

Tumor multiplicity For ethical reasons, any mouse that developed 10 SCCs or a tumor of 1 cm diameter was sacrificed. For statistical analysis, these mice were regarded as maintaining the number of tumors they had at sacrifice for the duration of the experiment. This only occurred in a small number of mice during the final 3 wk. Most of these mice were in the RA-treated UV-irradiated groups, and hence this assumption reduced the level of increase caused by the RA. Skh:HR-1 mice treated with RA and

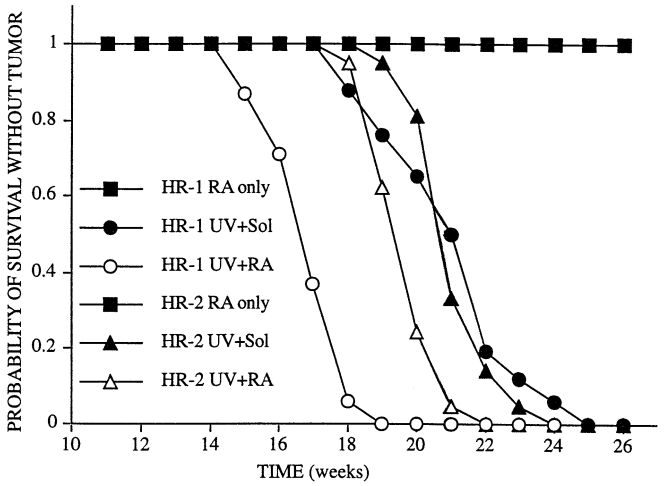

Figure 3. RA reduces the probability that a UV-irradiated mouse will survive without developing an SCC. Groups of 20 Skh:HR-1 and Skh:HR-2 mice were exposed to UVR and treated topically with RA or solvent (Sol) for 25 wk. $p < 0.001$ (Skh:HR-2 UV+RA) or 0.0001 (Skh:HR-1 UV+RA) compared with UV plus solvent treated controls of the same mouse strain [Logrank (Mantel–Cox) test].

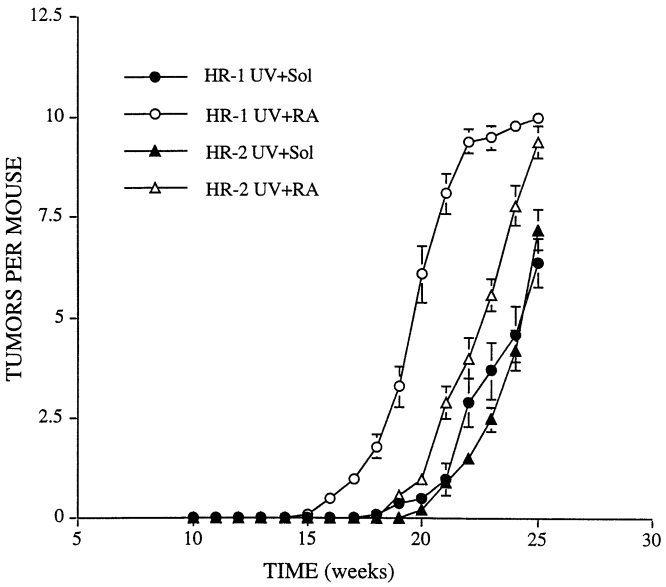

Figure 4. RA increases the number of UV-induced SCCs per mouse. Groups of 20 Skh:HR-1 and Skh:HR2 mice were exposed to UVR and treated topically with RA or solvent (Sol) for 25 wk. $p < 0.001$ for UV+RA mice compared with UV+Sol treated mice of the same strain, at every time point from week 16 for Skh:HR-1 mice and at every time point from week 21 for Skh:HR-2 mice (analysis of variance). Mean and SEM are shown; where the SEM cannot be seen, it is too small to be obvious.

UVR had significantly larger numbers of tumors than Skh:HR-1 mice treated with solvent and UVR at all time points from week 16 onwards (**Fig 4**). Skh:HR-2 mice which received RA and UVR also had significantly higher tumor counts than Skh:HR-2 mice treated with solvent and UVR at all time points from week 21 onwards. There were no significant differences between solvent plus UV irradiated Skh:HR-1 and Skh:HR-2 mice, whereas RA plus UVR treated Skh:HR-2 mice had significantly fewer tumors ($p < 0.001$) than similarly treated Skh:HR-1 mice at all times from week 15 onwards. Thus RA increased tumor multiplicity, and the light tan of the irradiated Skh:HR-2 mice did not protect, but the darker tan of the RA plus UVR treated Skh:HR-2 mice did provide some protection.

Tumor histology Examples of tumors from each mouse group were paraffin embedded for routine histologic analysis; four to nine tumors were examined per group. All tumors in each group were histologically confirmed to be SCCs. In the mice treated with RA and UVR, the treated skin between the tumors appeared hyperplastic and hyperkeratotic (**Fig 2**); hence this was also biopsied for histologic analysis. It was found to be carcinoma *in situ*. Thus, in addition to the increased number of clinically detectable SCCs in RA-treated mice, there were signs of increased malignant transformation in skin between these lesions.

DISCUSSION

These experiments show that topical application of 0.05% RA followed by exposure to one-third MED of solar-simulated UV radiation the following day (to mimic application of RA at night) enhances SCC formation. This occurred in both albino and congenic lightly pigmented mice. The light tan of the pigmented mice did not protect from photocarcinogenesis, but comparison of UVR plus RA treated Skh:HR-1 and Skh:HR-2 mice showed that the dark tan of the Skh:HR-2 mice did provide protection from carcinogenesis. This protection was not able to overcome the enhancing effect of the RA, however, therefore the development of a relatively dark tan only provides modest protection from UVR-induced carcinogenesis. This is consistent with a previous report that UVR-induced tanning provides some protection from DNA damage in skin types III and IV but not in types I and II (Young *et al*, 1991).

We have previously shown that RA augments UVR-induced melanogenesis in Skh:HR-2 mice (Ho *et al*, 1992). In these experiments melanin was stained by the Masson–Fontana method and quantitated by image analysis. In further studies it was shown that RA more than doubled the melanin concentration in UV-irradiated skin, and that the RA increased melanocyte activation (Welsh *et al*, 1999). In the studies described now, skin pigmentation was monitored without sacrificing the mice at different time points by measuring a melanin index by reflectance spectroscopy, with similar results to what we have reported previously. Previously we have studied the effects of RA on pigmentation for up to 6 w; the studies reported here show that the increased pigmentation was maintained for 24 w. In this study the reflectance spectrometer readings of the Skh:HR-1 mice rose at about 10–15 wk in response to UVR plus RA. This was not caused by increased melanin production as these mice are genetically unable to produce melanin, and did not appear visibly pigmented. It was most probably caused by the increased skin thickness and hyperkeratosis which commenced at this stage, due to the large number of solar-keratosis-like lesions and developing SCCs.

The first report that RA can enhance photocarcinogenesis was by Epstein (Epstein, 1977). In these experiments mice were exposed to 1380 mJ per cm^2 UVR from a high pressure mercury arc source three times per wk and treated with a high RA dose 0.3%) after UV exposure. We also treated with RA after UV, but used a much lower RA dose. Subsequent studies using 1.25 mJ per cm^2 UVB followed by lower doses of RA showed that 0.05% RA inhibited UVR-induced SCC induction, whereas lower doses were without effect (Epstein and Grekin, 1981). This is the same RA dose as used in our study, but the opposite result was obtained. In the studies by Epstein and Grekin the antioxidant BHT was added to the solvent, and therefore it is possible that an interaction between BHT and RA was required for the inhibition of photocarcinogenesis. Another difference between our studies is the UV source; they used a high pressure mercury arc emitting between 280 and 320 nm whereas we used solar-simulated UVR.

RA has also been reported to enhance photocarcinogenesis induced by 2 h exposure to a xenon arc solar-simulator (Forbes *et al*, 1979). The UVR doses were not given, but they were equated to 5 min of noon summer sun, which is within the region of the doses used in our study. In these experiments lower (0.001%) and higher (0.01%) RA doses than used in our study were effective. As in our study, the RA was applied daily after the UVR, and the skin tumors induced were SCCs or carcinoma *in situ*. Thus this is consistent with our observations.

In another study, mice were exposed to the equivalent of six times the human MED from FS20 sun lamps which emit UVA, UVB, and UVC (Kligman and Kligman, 1981). RA (0.001% or 0.01%) applied after each UV irradiation or following tumor induction did not affect carcinogenesis in Skh:HR-2 mice. In experiments where 0.001% RA was commenced after tumor formation, however, the regression rate increased. Using a different experimental protocol, where mice were irradiated with daily doses of 1.5 MED solar-simulated UVR for 10 wk and then treated with 0.05% RA for 30 wk after completion of the irradiation regime, there was no difference between the base cream and RA (Kligman and Crosby, 1996). Mice which were exposed to 3 MED per day UV emitted by FS40 sunlamps (Connor *et al*, 1983) had reduced photocarcinogenesis when treated with 3.4 nmol RA applied after each UV dose. In these studies the tumors were a mixture of benign papillomas and SCCs. FS40 sunlamps emit mainly UVB, but are contaminated with lower wavelengths than are found in sunlight. Thus these studies, which obtained different results to ours, either did not use solar-simulated UVR or used UV doses above the MED. It appears possible that topical RA may not enhance photocarcinogenesis induced by UV doses above the erythemal threshold or with non-solar-simulated spectra. The reason for this is unclear, but it is possible that in these situations tumor induction may be too rapid to be augmented.

A recent study by Wang *et al* (1999) highlighted the complex interactions between UV and RA by showing that pre-treatment of human skin with RA reduced the loss of nuclear retinoid receptors that occurred upon UV exposure. The significance of this with respect to carcinogenesis is unknown.

Despite increasing SCC induction and causing melanocyte activation (Welsh *et al*, 1999), RA in association with UVR did not induce melanoma formation in these experiments. Thus our data indicate that using this regime of 0.05% RA followed daily by a period of dark, and then low dose (0.33 MED) solar-simulated UVR, RA enhances SCC induction. This model was based on how humans commonly use topical RA for a number of dermatologic conditions, and indicates that topical RA should be used with caution.

We would like to thank Dr. Malcolm France, Department of Veterinary Anatomy and Pathology, University of Sydney, for histological analysis of the tumors. We would also like to thank the Leo and Jenny Leukaemia and Cancer Foundations of Australia and the University of Sydney Cancer Research Fund for financial support.

REFERENCES

Bestak R, Barnetson RStC, Nearn MR, Halliday GM: Sunscreen protection of contact hypersensitivity responses from chronic solar-simulated ultraviolet irradiation correlates with the absorption spectrum of the sunscreen. *J Invest Dermatol* 105:345–351, 1995

Chen L-C, De Luca LM. In: Mukhtar H (ed). *Skin Cancer: Mechanisms and Human Relevance*. Boca Raton, FL: CRC Press, 1995, pp. 401–424

Connor MJ, Lowe NJ, Breeding JH, Chalet M: Inhibition of ultraviolet-B skin carcinogenesis by all-*trans*-retinoic acid regimes that inhibit ornithine decarboxylase induction. *Cancer Res* 43:171–174, 1983

Damian DL, Halliday GM, Barnetson RStC: Prediction of minimal erythema dose with a reflectance melanin meter. *Br J Dermatol* 136:714–718, 1997

English DR, Armstrong BK, Kricker A, Fleming C: Sunlight and cancer. *Cancer Causes & Control* 8:271–283, 1997

Epstein JH: Chemicals and photocarcinogenesis. *Aust J Dermatol* 18:57–61, 1977

Epstein JH, Grekin DA: Inhibition of ultraviolet-induced carcinogenesis by all-*trans* retinoic acid. *J Invest Dermatol* 76:178–180, 1981

Forbes PD, Urbach F, Davies RE: Enhancement of experimental photocarcinogenesis by topical retinoic acid. *Cancer Lett.* 7:85–90, 1979

Ho KKL, Halliday GM, Barnetson RStC: Topical retinoic acid augments ultraviolet light-induced melanogenesis. *Melanoma Res* 2:41–45, 1992

Kligman LH, Crosby MJ: Topical tretinoin enhances corticosteroid-induced inhibition of tumorigenesis in hairless mice previously exposed to solar simulating radiation. *Cancer Lett.* 107:217–222, 1996

Kligman LH, Kligman AM: Lack of enhancement of experimental photocarcinogenesis by topical retinoic acid. *Arch Dermatol Res* 270:453–462, 1981

Reeve VE, Greenoak GE, Boehm-Wilcox C, Canfield PJ, Gallagher CH: Effect of topical 5-methoxypsoralen on tumorigenesis induced in albino and pigmented hairless mouse skin by UV irradiation. *J Photochem Photobiol B—Biology* 5:343–357, 1990

Wang Z, Boudjelal M, Kang S, Voorhees JJ, Fisher GJ: Ultraviolet irradiation of human skin causes functional vitamin A deficiency, preventable by all-*trans* retinoic acid pre-treatment. *Nature Med* 5:418–422, 1999

Welsh BM, Mason RS, Halliday GM: Topical all-*trans* retinoic acid augments ultraviolet radiation-induced increases in activated melanocyte numbers in mice. *J Invest Dermatol* 112:271–278, 1999

Young AR, Potten CS, Chadwick CA, Murphy GM, Hawk JLM, Cohen AJ: Photoprotection and 5-MOP photochemoprotection from UVR-induced DNA damage in humans: the role of skin type. *J Invest Dermatol* 97:942–948, 1991